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Title: An oxalate efflux transporter from the brown-rot fungus *Fomitopsis palustris*

Running title: Oxalate transporter from *Fomitopsis palustris*

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Abstract

An oxalate-fermenting brown-rot fungus, *Fomitopsis palustris*, secretes large amounts of oxalic acid during wood decay. Secretion of oxalic acid is indispensable for the degradation of wood cell walls, but almost nothing is known about the transport mechanism by which oxalic acid is secreted from *F. palustris* hyphal cells. We characterized the mechanism for oxalate transport using membrane vesicles of *F. palustris*. Oxalate transport in *F. palustris* was ATP dependent and was strongly inhibited by several inhibitors such as valinomycin and NH_4^+, suggesting the presence of a secondary oxalate transporter in this fungus. We then isolated a cDNA, *Fomitopsis palustris* Oxalic Acid Resistance (*FpOAR*), from *F. palustris* by functional screening of yeast transformants with cDNAs grown on oxalic acid-containing plates. FpOAR is predicted to be a membrane protein that possesses six transmembrane domains, but shows no similarity with known oxalate transporters. The yeast transformant possessing *FpOAR* (*FpOAR*-transformant) acquired resistance to oxalic acid and contained less oxalate than the control transformant. Biochemical analyses using membrane vesicles of the *FpOAR*-transformant showed that the oxalate transport property of FpOAR was consistent with that observed in membrane vesicles of *F. palustris*. The quantity of *FpOAR* transcripts was correlated with increasing oxalic acid accumulation in the culture medium and was induced when exogenous oxalate was added to the medium. These results strongly suggest that FpOAR plays an important role in wood decay by acting as a secondary transporter responsible for secretion of oxalate by *F. palustris*.

Oxalic acid is produced by a wide variety of members of five kingdoms (*Monera*, *Protista*, *Fungi*, *Plantae*, and *Animalia*) (32), and plays multiple roles as a proton and electron source and strong metal chelator in ecosystem processes (11). With regard to wood decay by brown-rot fungi, which cause severe damage to wooden structures, oxalic acid is secreted by the fungus in large amounts (8, 38). Several important roles of oxalic acid in the brown-rot decay process are proposed. Oxalic acid hydrolyzes side-chains of hemicelluloses, then depolymerizes the hemicellulose backbones and amorphous cellulose, thus increasing the porosity of the wood structure to the hyphal sheath, decay enzymes, or other low-molecular-weight decay agents (12). In cellulose degradation by the Fenton reaction, a low concentration of oxalate promotes degradation (41) by facilitating hydroxyl radical formation (45), but a higher concentration of the acid inhibits the degradation (41) and radical formation (45). Furthermore, oxalate forms Fe-oxalate complexes, which can then diffuse into the wood cell wall for the Fenton reaction by which oxalate protects the hyphae of brown-rot fungi from attack by the Fenton reagent (2, 16, 38). Therefore, from the viewpoint of preservation of wooden buildings and cultural treasures, elucidation of the biochemical mechanisms for oxalate biosynthesis and its secretion in brown-rot fungi is needed.

Recently, we clarified the mechanisms for oxalate biosynthesis in the brown-rot basidiomycete *Fomitopsis palustris*, which is used as a standard fungus for Japanese Industrial Standards decay resistance tests. *Fomitopsis palustris* secretes large amounts of oxalate (33–78 mM) into the culture fluid, which lowers the medium pH to ca. 2. The terminal enzymes for oxalate biosynthesis are cytosolic oxaloacetate acetylhydrolase

(OAAH, EC 3.7.1.1) (1) and peroxisomal cytochrome *c* dependent glyoxylate dehydrogenase (15, 43). Our biochemical analysis of oxalate fermentation by *F. palustris* led to the conclusion that the fungus acquires energy for growth by oxidizing glucose mainly to oxalate through the tricarboxylic acid (TCA) and glyoxylate (GLOX) cycles (28, 29, 35).

Importantly, it is widely recognized that oxalate is toxic to organisms. Exogenously added oxalate inhibits sporulation and growth of the filamentous fungi *Fusarium oxysporum* (7) and *Pythium vexans* (46). Therefore, oxalate-producing brown-rot fungi must have a mechanism to prevent damage caused by intra- and extra-cellular oxalate. Several brown-rot fungi show not only oxalate-producing but also oxalate-degrading activity. Among these oxalate-producing and -degrading brown-rot fungi, *Postia placenta* produces oxalate decarboxylase to convert oxalate to formate and CO_2 (25), which prevents overaccumulation of oxalic acid and forms a non-toxic, buffered, low-pH environment that facilitates the brown-rot decay process (26). *Gloephyllum trabeum* degrades extracellularly added oxalate to give rise to CO_2 (10). Similarly, *Fomitopsis pinicola* and *Meruliporia incrassata* have been reported to decrease the amount of extracellular oxalate (36).

In contrast to these oxalate-producing and -degrading brown-rot fungi, oxalate-decomposing activity has not been reported for *F. palustris*. However, *F. palustris* grows vigorously in the presence of a high oxalate concentration (28, 29). The marked accumulation of oxalic acid in the culture fluid of *F. palustris* suggests that the fungus has an efficient system to transport oxalate out of the cells. Oxalate is continuously biosynthesized as a major end product of primary metabolism in the cytosol and

peroxisome of *F. palustris* (15, 28, 35). Thus, while transporting oxalate from the peroxisome to the cytosol and eventually out of the cells, essential metabolic processes should be protected from oxalate toxicity. In this context, the oxalate transporter in the cytosolic membrane is expected to reduce the intracellular concentration of oxalate, which probably contributes to the oxalate resistance system in *F. palustris*. However, almost nothing is known about the transport systems of *F. palustris*.

Oxalate transporters are known to play several important roles in metabolizing or excreting oxalate by other organisms. For example, *Oxalobacter formigenes*, a gram-negative anaerobe, possesses the oxalate:formate exchange protein OxlT, which is essential for *O. formigenes* to produce ATP (21). Humans and mice possess the SLC26 multifunctional anion exchangers, of which SLC26A6 (humans) and Slc26a6 (mice) are proposed to exchange Cl^- and SO_4^{2-} for oxalate or formate in the intestinal villi, renal proximal tubule and cardiac myocytes (27). Furthermore, hepatopancreatic lysosomal membrane vesicles isolated from the lobster *Homarus americanus* exchange oxalate for Cl^- in relation to Zn^{2+} detoxification (40).

Recently, Mch5, a homolog of a putative oxalate:formate antiporter from *Aspergillus fumigatus*, was postulated to be a putative oxalate transporter in yeast *Saccharomyces cerevisiae*. However, the oxalate-transporting activity of Mch5 and the possible roles of the gene product have not been demonstrated (5). Accordingly, an oxalate-transporting protein has not previously been characterized experimentally from any fungus.

Here we describe a cDNA encoding the protein involved in oxalate transport in *F. palustris*. To isolate a cDNA encoding the oxalate transporter, we previously isolated

fungal cDNAs from yeast transformants with cDNA of *F. palustris* grown in the presence of a high oxalate concentration that is lethal to wild-type cells (49). Even if oxalate was incorporated into the cells, the transformants probably transported oxalate out of the cells or it was degraded by the gene products of the cDNA from *F. palustris*. Accordingly, these cDNAs were expected to encode transporters catalyzing oxalate transport out of the cells or oxalate-decomposing enzymes and other proteins possessing unidentified functions. By this strategy, we successfully isolated the cDNA *FpTRP26* conferring oxalic acid resistance for *F. palustris* (49). We have further characterized the remaining transformants showing oxalic acid resistance (49) and obtained the gene product, FpOAR (*Fomitopsis palustris* Oxalic Acid Resistance), which is a putative plasma membrane protein that showed distinct oxalate transport activity in yeast membrane vesicles. The oxalate transport property of FpOAR was similar to that of *F. palustris* membrane vesicles. These results strongly suggest that FpOAR is involved in oxalate secretion in *F. palustris* hyphal cells.

MATERIALS AND METHODS

***Fomitopsis palustris* culture conditions.** Two mycelial plugs (5 mm in diameter) of *F. palustris* (Berkely et Curtis) Murill TYP6137 were grown as a stationary culture in a 200 ml Erlenmeyer flask containing 40 ml liquid medium as previously reported (49).

Cloning of *F. palustris* cDNA conferring oxalic acid resistance. Functional screening of a *Saccharomyces cerevisiae* AD12345678 (6) transformant with a *F. palustris* cDNA library with vector pDR196 (51) was carried out to screen the transformants to grow with

12 mM oxalic acid (49). Because the cDNA fragment recovered from oxalic acid-resistant yeast showing a strong phenotype lacked the 5’ region, the 5’ end of the cDNA was determined with the Gene Racer™ kit (Invitrogen) according to the manufacturer's instructions. The 5’ end of the cDNA was cloned with the gene-specific antisense PCR primer 5'-CCACGACCACCGCCGCAAGCATGAAGA-3'. The amplified cDNA fragments were subcloned into the TA cloning vector with the pCR2 TOPO TA cloning kit (Invitrogen). A clone containing an insert of the expected size was sequenced. The open reading frame (ORF) of the cDNA was cloned with the PCR primers 5'-ACTAGTATGACCGACCTGCATCGAAG-3' (sense) and 5'-GGATCCTCAGAGAAGATCTTCTTGCCG-3' (antisense), which were gene-specific primers containing *Spe* I and the *Bam*HI restriction site, respectively. The coding region thus obtained was named *FpOAR* (*Fomitopsis palustris* Oxalic Acid Resistance).

Characterization of oxalic acid-resistance activity of FpOAR. The plasmid containing *FpOAR* was reintroduced into the *S. cerevisiae* AD12345678 strain to characterize its oxalic acid resistance according to our method reported previously (49). Oxalic acid resistance was determined by growth of the yeast transformant. Similarly, the transformants were cultivated separately on SD (–Ura) plates containing different HCl concentrations (pH 1.5, 1.6, and 2.2).

Quantification of oxalic acid. Oxalic acid in the *F. palustris* culture medium was quantified with a commercial kit after the pH of the medium was adjusted for the assay (Roche, Germany) (48), while GC-MS analysis (14) was conducted for that in yeast cells. Yeast (OD_{600} 0.1) was cultured at 30°C in 50 ml SD (–Ura) liquid medium containing 2

150 mM oxalic acid until OD_{600} 1.0–2.0. The cells were harvested by centrifugation at 1000×*g*
151 for 10 min and washed twice with cold distilled water. The dry weight of cells was
152 determined after freeze-drying for 5 h. To the dried cells, 250 µl 1*N* HCl and 600 µl
153 ethylacetate were added and cells were homogenized with glass beads (Toshinriko, No. 04)
154 for 4 min. The oxalic acid extracted with ethylacetate was quantified as previously
155 described (14).

156 GC-MS (EI) was performed on a Shimadzu GC-MS QP-5050A. The column conditions
157 were as follows: CBP1-M25-025, 25 m × 0.22 mm (i.d.) (Shimadzu), column temperature
158 80–240°C (8°C/min), carrier gas He, and carrier gas flow rate 0.8 ml/min.

159 **Quantitative real-time PCR analysis of gene transcription.** Total RNA was isolated
160 from *F. palustris* mycelia with the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA
161 synthesis was performed with Superscript II reverse transcriptase (Invitrogen) using 0.2 µg
162 RNA. Real-time quantitative PCR was performed with a 7300 Real Time System (Applied
163 Biosystems) and amplicons were detected with SYBR Green (Applied Biosystems).
164 Quantifications of the amplicons were based on standard curves prepared for each target
165 cDNA. The gene-specific primers 5‘-CCTCGAACAAGCGAATTCTCTTT-3’ and
166 5’-AGTGTCCCGCCGAGGAA-3’ were used to generate an 85 bp amplicon for *FpOAR*
167 transcripts. The amount of transcripts was normalized by comparison with those of a 75 bp
168 amplicon derived from either total RNA or 28S rRNA (GenBank accession no. AY515333)
169 with the primers 5'-TGACACGGACTACCAGTGCTTT-3' (sense) and
170 5'-CACCCATTTTGAGCTGCATTC-3' (antisense).

FpOAR transcripts were quantified in mycelia from cultures to which either oxalate (50 mM final concentration) or H_2O was added on day 3. The cultures were incubated for 12 h prior to RNA extraction.

Preparation of *F. palustris* membrane vesicles. The membrane vesicles were prepared from *F. palustris* hyphal cells as described previously (33). Cells were collected and homogenized in ice-cold homogenizing buffer (0.1 M Tris-HCl, pH 8.0, 10% [v/v] glycerol, 5 mM EDTA; 2 ml/g fresh weight of mycelia) using a Phoenix blender (USA). Prior to use, KCl, DTT, and PMSF were added to the autoclaved buffer at the final concentrations 150 mM, 3.3 mM and 1 mM, respectively, and the mixture was stirred for 1 min. Unbroken cells and debris were removed by centrifugation twice at 8,000×*g* for 10 min. The supernatants were pooled and centrifuged at 100,000×*g* for 60 min. The microsome fraction was resuspended with autoclaved resuspension buffer (10 mM Tris-HCl, pH 7.6, 10% [v/v] glycerol, 1 mM EDTA). Isolated membrane vesicles were stored at –80°C in resuspension buffer containing 1 mM DTT and 1 mM PMSF until use.

Yeast membrane vesicles for *in vitro* transport studies were isolated as described previously (17, 44) with the following modifications. The *S. cerevisiae* AD12345678 strain was grown overnight in SD (–Ura) liquid medium to OD_{600} 1–2. Cells were washed twice with water and resuspended to OD_{600} 1–2 in spheroplast buffer (1.1 M sorbitol, 20 mM Tris-HCl [pH 7.6], 1 mM DTT containing 57 units/ml Zymolyase 20T). After cell wall digestion was completed, spheroplasts were collected by centrifugation at 1200×*g* for 10 min. Cell lysis was performed gently on ice in 25 ml breaking buffer (1.1 M glycerol, 50 mM Tris-ascorbate [pH 7.4], 5 mM EDTA, 1 mM DTT, 1.5% polyvinylpyrrolidone, 2

193 mg/ml BSA, 1 mM PMSF, 10 µg/ml leupeptin, 2 µg/ml aprotinin, and 2 µg/ml pepstatin)
194 with a Dounce homogenizer and 40 strokes with a tight-fitting glass piston. Unbroken cells
195 and debris were removed by centrifugation twice at 3,000×*g* for 10 min. The supernatants
196 were pooled and centrifuged at 100,000×*g* for 60 min. Microsomal membrane proteins
197 were quantified by Bradford's method (3). The pellet was suspended to a protein
198 concentration of about 5 mg/ml in vesicle buffer (1.1 M glycerol, 50 mM Tris-MES [pH
199 7.4], 1 mM EDTA, 1 mM DTT, 2 mg/ml BSA, 1 mM PMSF, 1 µg/ml leupeptin, 2 µg/ml
200 aprotinin, and 2 µg/ml pepstatin). Small aliquots were immediately used for transport
201 assays or stored at –80°C until use. Membrane vesicles prepared with this method are a
202 mixture of inside-out and right-side-out orientation, whereas only inside-out vesicles can
203 hydrolyze ATP owing to the outside orientation of the ATPase catalytic sites that drive
204 membrane transport and because ATP cannot permeate the membrane. Therefore, in this
205 experiment the observation of oxalate uptake into the inside-out vesicle indicates oxalate
206 efflux activity of the hyphal cell membrane.

207 ***In vitro* oxalate transport assay.** Vesicles (100 µg protein) were mixed with transport
208 buffer (0.4 M glycerol, 100 mM KCl, 20 mM Tris-MES [pH 7.4], 1 mM DTT) and
209 incubated with 0.2 mM [^{14}C]oxalic acid at 25 °C for 10 min in the absence or presence of 5
210 mM MgATP with a final volume of 125 µl, unless stated otherwise. In the assay using *F.*
211 *palustris* vesicles, the vesicles (500 µg protein) were suspended in a final volume of 500 µl.
212 Uptake of [^{14}C]oxalic acid (185 MBq/mmol) into membrane vesicles was measured by two
213 methods. For *F. palustris* membrane vesicles, 130 µL reaction mixture was loaded on a
214 Sephadex (G-50 fine) spin column and centrifuged at 2,000 rpm for 2 min. The

radioactivity of 100 µL filtrate was determined with a liquid scintillation counter. The yeast vesicles were absorbed onto nitrocellulose filters (3 cm in diameter, 0.45 µm pore size; GE Healthcare) by filtration with an aspirator. The vesicles on the membrane were washed three times with 1 ml transport buffer by filtration with an aspirator, dried on filter paper and subjected to liquid scintillation counting. Counts were corrected for background and quenching. Quantification and calculations were performed using a Liquid Scintillation Analyzer Tri-Carb 2800TR (Perkin Elmer, USA).

Effects of inhibitors on oxalate transport. Using *F. palustris* vesicles, the following inhibitors were added separately to assay solutions: 1 mM vanadate, 2 µM valinomycin, 1 mM NH_4Cl, 150 µM glibenclamide, or 5 µM cyclosporin A. Oxalate transport was recorded as described above.

To assess the inhibition using yeast vesicles from the *FpOAR*-transformant, we used 5 µM verapamil, and 5 µM gramicidin D in addition to the reagents used for the *F. palustris* vesicle assay. Oxalate transport was recorded as described above.

Statistical analysis. Analyses were carried out using ANOVA followed by the Dunnett test with a level of significance of $p = 0.05$.

RESULTS

Biochemical analysis of oxalate transport using *F. palustris* membrane vesicles. To characterize oxalate transport of *F. palustris* we prepared membrane vesicles from hyphae of the fungus and investigated whether MgATP is required for the transport activity. In the

presence of MgATP, [^{14}C]oxalate uptake into the vesicles was 4.27 times that in the control lacking MgATP (Fig. 1). This result indicated that MgATP is needed for oxalate transport activity in *F. palustris*.

We then investigated the effects of a variety of inhibitors on oxalate transport. Vanadate, which is an inhibitor of P-type ATPases and ATP-binding cassette (ABC) transporters, reduced oxalate uptake into the membrane vesicle by 68.0%. The addition of valinomycin or NH_4Cl, which abolish the $\Delta\Psi$ and ΔpH across the membrane, respectively, inhibited oxalate uptake by 86.3% and 90.1%, respectively. In contrast, glybencleamide and cyclosporin A, which are typical inhibitors of ABC transporters, did not influence oxalate transport. These results indicate that ABC transporters are not primarily responsible for oxalate transport. Collectively, the results strongly suggest that a secondary oxalate transporter functions in *F. palustris*, in which $\Delta\Psi$ and ΔpH are involved.

Cloning of a cDNA conferring oxalic acid resistance on the yeast transformant. Previously, we isolated the cDNA FpTRP26 from one of eight *S. cerevisiae* transformants (49). From the remaining transformants, we isolated one cDNA (1170 bp), named *FpOAR* (GenBank accession no. AB372882), which was found to encode a deduced 42,873 Da protein. A BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/) search revealed that the deduced FpOAR showed 86%, 82%, 73%, 66% and 43% identities with predicted membrane proteins of *Postia placenta* (24) and *Phanerochaete chrysosporium* (23), a major intrinsic protein of *Laccaria bicolor* (22), a hypothetical protein CC1G_06363 of *Coprinopsis cinerea* Okayama 7#130 (4), and a transmembrane protein of *Cryptococcus neoformans* var. *neoformans* JEC21 (19), respectively (Fig. 2). These are all basidiomycete

fungi and, in particular, *P. placenta* and *P. chrysosporium* are brown- and white-rot fungi, respectively. However, no biochemical functions have been elucidated for these proteins to date. A close similarity between FpOAR and oxalate-degrading enzymes, such as oxalate decarboxylase (EC 4.1.1.2) and oxalate oxidase (EC 1.2.3.4), was not observed. However, the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/) predicted that FpOAR possesses six transmembrane domains (Supplemental Fig. S1).

Oxalic acid resistance in yeast transformants with the cDNA FpOAR. On plates containing 8.5–10 mM oxalic acid, the yeast transformant possessing the cDNA *FpOAR* (*FpOAR*-transformant) showed clear cell growth, whereas no growth was observed in the control transformant with an empty vector (Figs. 3*A*, *B*). At an oxalic acid concentration below 8.5 mM, the growth of the control and *FpOAR*-transformant did not differ significantly (data not shown). In medium with the same pH containing malonate but not oxalate, no difference between the control and *FpOAR*-transformant was observed, showing that oxalate is more toxic than malonate (Fig. 3*C*). To eliminate the possibility that the *FpOAR*-transformant was resistant to low pH (2.2–1.5), we investigated the growth of the *FpOAR*-transformant on a low-pH plate (Fig. 3*D*), but no difference in growth between the control and *FpOAR*-transformant was observed at pH 2.2-1.5 and pH 5. We then compared the oxalic acid contents of the *FpOAR*-transformant and the empty vector control grown in the presence of 2 mM oxalic acid. No difference in cell growth was observed between the two cultures at this concentration, but the cellular content of oxalate in the *FpOAR*-transformant strongly decreased to 25% compared with that of the control (Fig. 4).

Oxalate transport in vesicles of the *FpOAR*-transformant. In the presence of

MgATP, membrane vesicles of the *FpOAR*-transformant significantly accumulated [^{14}C]oxalate, which was 1.4 times greater than that of the control lacking MgATP. When the assay was conducted at 4 °C or with vesicles denatured at 95 °C, oxalate transport did not differ from that of the empty vector control (Fig. 5).

Vanadate and gramicidin D, which are inhibitors of P-type ATPases and a monovalent-selective ionophore that dissipates both the pH gradient and membrane potential, inhibited oxalate transport by 28.5% and 55.8%, respectively (Fig. 6). Valinomycin and NH_4Cl, which abolish $\Delta\Psi$ and ΔpH across the membrane, respectively, inhibited oxalate transport by 34.6% and 34.9%, respectively. In contrast, the inhibitors of ABC transporters, glybencleamide and verapamil, slightly inhibited and did not significantly inhibit, respectively, oxalate transport in *FpOAR*-transformant vesicles. These results are in agreement with the oxalate-transporting activity exhibited by membrane vesicles of *F. palustris* (Fig. 1), and suggest that FpOAR is directly involved in oxalate secretion.

Quantitative real-time PCR analysis of *FpOAR* transcripts in *F. palustris*. The quantity of *FpOAR* transcripts increased concomitantly with accumulation of oxalate in the culture medium (Figs. 7*A*, *B* and *C*). High levels were maintained even in the stationary phase during days 9–13, while oxalic acid accumulation increased (day 13, 34 mM). The amount of *FpOAR* transcripts increased three-fold compared with that of the control when 50 mM oxalic acid was added to the medium (Fig. 8).

DISCUSSION

FpOAR functions as a secondary oxalate transporter conferring oxalic acid resistance in *F. palustris.* In this study, we characterized oxalate transport using membrane vesicles of *F. palustris.* We isolated a cDNA, *FpOAR*, encoding a novel membrane oxalate transporter protein specifically conserved among basidiomycete fungi.

The isolated cDNA *FpOAR* conferred resistance to oxalic acid on the *FpOAR*-transformant (Figs. 3*A*, *B*, *C* and *D*). The oxalate transport activity in the membrane vesicles of the yeast (Fig. 5) indicated that FpOAR was responsible for oxalate transport. The effects of inhibitors on oxalate transport in membrane vesicles of the *FpOAR*-transformant (Fig. 6) suggest that FpOAR transports oxalate by a secondary transport system, in which ΔpH and ΔΨ are the driving force. The effects of the inhibitors on yeast vesicles (Fig. 6) were similar to those on vesicles from *F. palustris* (Fig. 1). Furthermore, expression of *FpOAR* and oxalic acid accumulation in the medium were positively correlated (Fig. 7*A*, *B* and *C*). Expression of *FpOAR* was induced by addition of oxalic acid to the culture (Fig. 8). Collectively, these results strongly suggest the involvement of FpOAR as a secondary oxalate transporter to confer oxalic acid resistance in *F. palustris*.

FpOAR is a plasma membrane-localized novel secondary oxalate transporter. FpOAR is proposed to be a novel oxalate secondary transporter based on three lines of evidence: 1) MgATP was needed for oxalate transport activity; 2) FpOAR does not possess an ATP-hydrolyzing domain (Walker motifs and ABC signature) as in ABC transporters, which function as primary transporters (47); and 3) there is no similarity between FpOAR and known oxalate transporters, e.g. SLC26 family proteins (27), the oxalate:formate

antiport protein, OxlT (21), and the putative yeast monocarboxylate transporter, Mch5 (5).

FpOAR is suggested to be a plasma membrane oxalate efflux transporter based on the lower oxalate content in the *FpOAR*-transformant compared with the control (Fig. 4). The probable cytosolic membrane localization of FpOAR is in agreement with the presence of six putative transmembrane domains in FpOAR (Supplemental Fig. S1). Furthermore, the PSORT program (http://psort.hgc.jp/) predicted that FpOAR is localized to the plasma membrane. The fungal plasma membrane H^+-ATPase generates a proton gradient through the cytosolic membrane (37), which is likely to drive oxalate transport through FpOAR.

FpOAR probably plays a crucial role to maintain carbon metabolism by *F. palustris*. During vegetative growth, *F. palustris* produces oxalate from a glucose carbon source with an 80% theoretical yield based on the amount of carbon in glucose (28). Two intrinsic features of *F. palustris* metabolism facilitate such a high yield of oxalate. First, the precursors of oxalate, glyoxylate and oxaloacetate, are constantly supplied through the GLOX cycle in the peroxisome, in which isocitrate lyase (FPICL1, EC 4.1.3.1) and malate synthase (EC 2.3.3.9) play indispensable roles as key enzymes (28, 29, 35). The FPICL1 and malate synthase of *F. palustris* show high activity even in glucose-grown mycelia (28, 29), which is in sharp contrast to the general GLOX cycle in other microorganisms, in which the functioning of the GLOX cycle is repressed by a glucose carbon source (35). Second, it is proposed that the GLOX cycle of *F. palustris* supplies succinate constitutively to the TCA cycle lacking 2-oxoglutarate dehydrogenase activity (28, 29, 35), by which oxaloacetate can be supplied for cytosolic oxalate production through the TCA cycle as well as the GLOX cycle (28, 29, 35).

Fomitopsis palustris continuously secretes oxalate as the fungus grows. This oxalate secretion is mainly due to continuous production of oxalate in primary carbon metabolism. Furthermore, the acid secretion could be due to the absence of oxalate degradation activity in *F. palustris*, in contrast to oxalate-decomposing white-rot fungi (9, 39, 48, 50) and several brown-rot fungi such as *P. placenta* (25) and *G. trabeum* (10). Moreover, we have suggested that *F. palustris* transports oxalate out of the cells to prevent possible inhibition of intracellular metabolic reactions. For example, oxalate might inhibit the activities of FPICL1 and malate synthase in peroxisomes of *F. palustris* (15, 35), because oxalate competitively inhibits the activities of the two enzymes *in vitro* (30, 31). In addition, oxalate produced from oxaloacetate by cytosolic OAAH in *F. palustris* (1, 35) possibly shows product inhibition for OAAH activity, because oxalate is a competitive inhibitor (*K*i = 19 μM) for OAAH from *Botrytis cinerea* (13). Therefore, the oxalate production coupled with energy metabolism and the efficient oxalate transport out of the cells are essential to maintain carbon metabolism in *F. palustris*. Aided by the oxalate export system, including FpOAR, and the oxalic acid resistance system, including FpTRP26 (49), carbon metabolism through the TCA and GLOX cycles are probably prevented from inhibition by oxalate *in vivo.* FpTRP26 was predicted to be a soluble protein and might help with oxalate export from the hyphal cells (49). The yeast transformant with FpTRP26 showed similar resistance to oxalic acid with regard to the acid concentration and pH (49). Further research is needed to elucidate how FpTRP26 and FpOAR cooperatively work in the oxalate resistance system. Whether the FpOAR homologous protein functions in oxalate-producing and -degrading brown-rot fungi requires investigation. Furthermore, in the same fungi, a

possible role of oxalate degradation in relation to energy metabolism should be evaluated to clarify the possible role of oxalate metabolism in brown-rot fungi. In this context, a possible ATP-generation by oxalate degradation is hypothesized for *P. placenta* (20) based on gene expression (24) as proposed for white-rot oxalate-degrading fungus *C. subvermispora* in which oxalate degradation by oxalate decarboxylase with formate dehydrogenase could produce NADH to be utilized for ATP synthesis (48).

FpOAR homologous protein is a potential target for development of new wood preservatives. The FpOAR homologous protein may be distributed widely in wood-rotting fungi, because the homologs showing significant identities with FpOAR were found in genomes of the brown- and white-rot fungi *P. placenta* and *P. chrysosporium*, respectively (Fig. 2). Oxalate production and secretion is commonly observed in wood-rotting fungi, although brown-rot fungi accumulate greater amounts of oxalate in the culture fluid than do white-rot fungi (8, 38). Therefore, the FpOAR homologous protein may generally function as an oxalate transporter in wood-rotting fungi. From the viewpoint of wood preservation, if the function of FpOAR is inhibited by wood preservatives, oxalate is expected to be accumulated intracellularly in brown-rot fungi and growth would be seriously damaged. By a similar inhibitory mechanism, oxalate excretion by white-rot fungi would decrease, which may repress lignin degradation, because a certain amount of excreted oxalate is important to stabilize Mn^{3+} oxidation for lignin degradation by manganese peroxidase (18). Accordingly, the FpOAR homologous protein would be a possible target protein to develop a new wood preservative to inactivate oxalate transport for extermination of wood-rotting fungi.

In summary, we have isolated a cDNA, *FpOAR*, encoding a novel oxalate transporter from *F. palustris*. The deduced FpOAR protein is suggested to play an important role in wood decay by acting as a secondary transporter responsible for secretion of oxalate from *F. palustris* hyphal cells. Two strategies for prevention of oxalate toxicity have been found for brown-rot fungi: 1) oxalate efflux through FpOAR; and 2) in addition to oxalate efflux, oxalate degradation in oxalate-producing and -degrading brown-rot fungi. Further characterization of oxalate transport by FpOAR is needed to elucidate the underlying mechanisms. Oxalate transport from the peroxisome to the cytosol remains to be investigated.

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The abbreviations used are: ABC, ATP-binding cassette; FpOAR, Fomitopsis palustris Oxalic Acid Resistance; GLOX, glyoxylate; TCA, tricarboxylic acid

612 **Figure legends**

613 FIG. 1. Oxalate transport and the effect of inhibitors using membrane vesicles from *F.*
614 *palustris*. Mean value ±standard deviation.

615 * $p < 0.05$ ($n = 3$).

616

617 FIG. 2. Neighbor-joining tree for FpOAR and its homologs. The tree was generated
618 using ClustalX (42) and visualized with TREEVIEW (34).
619 AAEY01000042, a transmembrane protein of *Cryptococcus neoformans* var.
620 neoformans JEC21 (GenBank accession no. AAEY01000042); gwh2.7.329.1, predicted
621 membrane proteins of *Phanerochaete chrysosporium* (JGI Protein ID 29288;
622 gwh2.7.329.1); Plus.C_740045, predicted membrane proteins of *Postia placenta* (JGI
623 Protein ID 111900; estExt_Genewise1Plus.C_740045); FpOAR (*Fomitopsis palustris*
624 Oxalic Acid Resistance) (GenBank accession no. AB372882); Lbscf0011g04660, a
625 major intrinsic protein of *Laccaria bicolor* (JGI Protein ID 297309;
626 eu2.Lbscf0011g04660); AACS01000187, a hypothetical protein CC1G_06363 of
627 *Coprinopsis cinerea* okayama 7#130 (GenBank accession no. AACS01000187).
628 Numbers at forks are bootstrap values derived from 1000 replicates. The scale bar
629 represents 0.05 substitutions per amino acid position.

630

631 FIG. 3. Growth of yeast transformants containing an empty vector (control) and *FpOAR*
632 on SD (–Ura) plates containing (A) no additives (standard), (B) oxalic acid, (C) malonic
633 acid and (D) HCl. Concentration of each additive and pH are shown above each image.
634 The number of yeast cells inoculated on the plate are indicated by ▶ at the top of
635 each figure (A-D).

636

637 FIG. 4. Amount of oxalic acid in yeast transformants containing an empty vector
638 (control) and *FpOAR*. Mean value ±standard deviation.** $p < 0.01$ ($n = 4$).

639

640 FIG. 5. Oxalate export in yeast transformants containing *FpOAR* (FpOAR-transformant)
641 and an empty vector (control). **$p < 0.01$ ($n = 4$). The complete system,
642 FpOAR-transformant + MgATP, showed significantly greater oxalate transport with **
643 than any other system. Denatured: the assay using the vesicles denatured at 95°C; 4°C:
644 the assay conducted at 4°C. Mean value ±standard deviation.

645

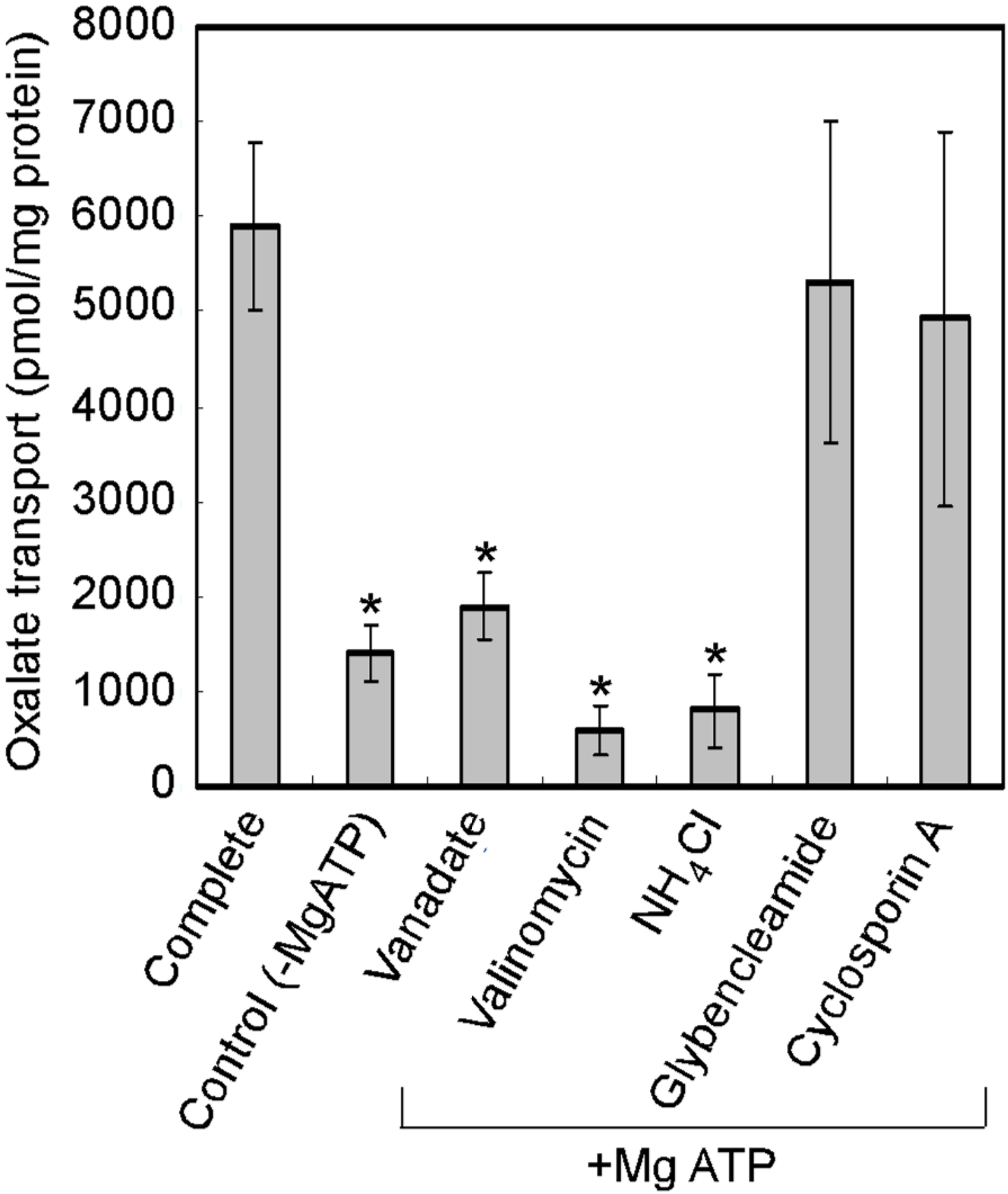
646 FIG. 6. Oxalate transport and effect of inhibitors using vesicles prepared from the
647 *FpOAR*-transformant. The reaction was carried out for 20 min. Mean value ±standard
648 deviation. * $p < 0.05$ ($n = 3$).

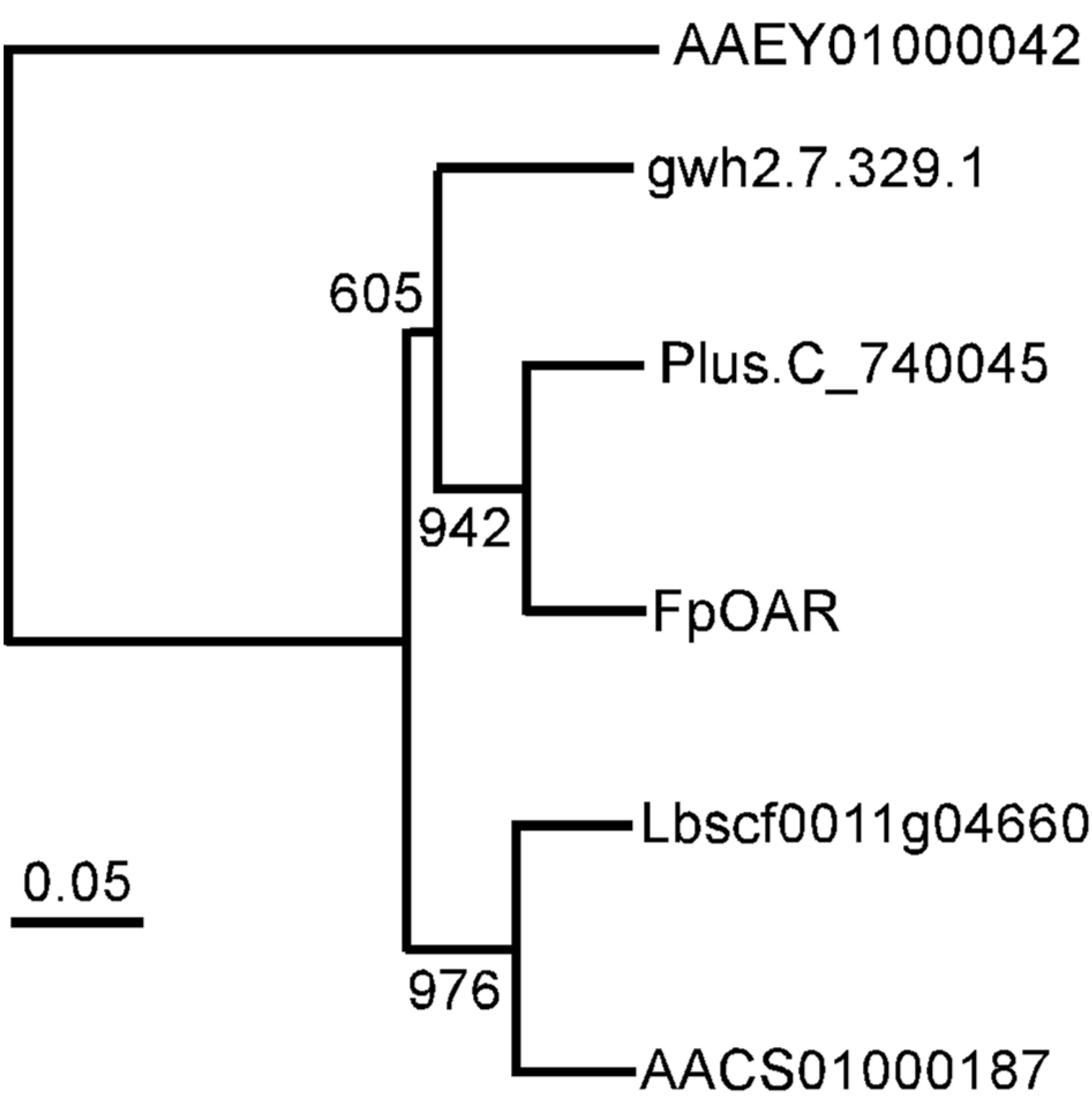
649

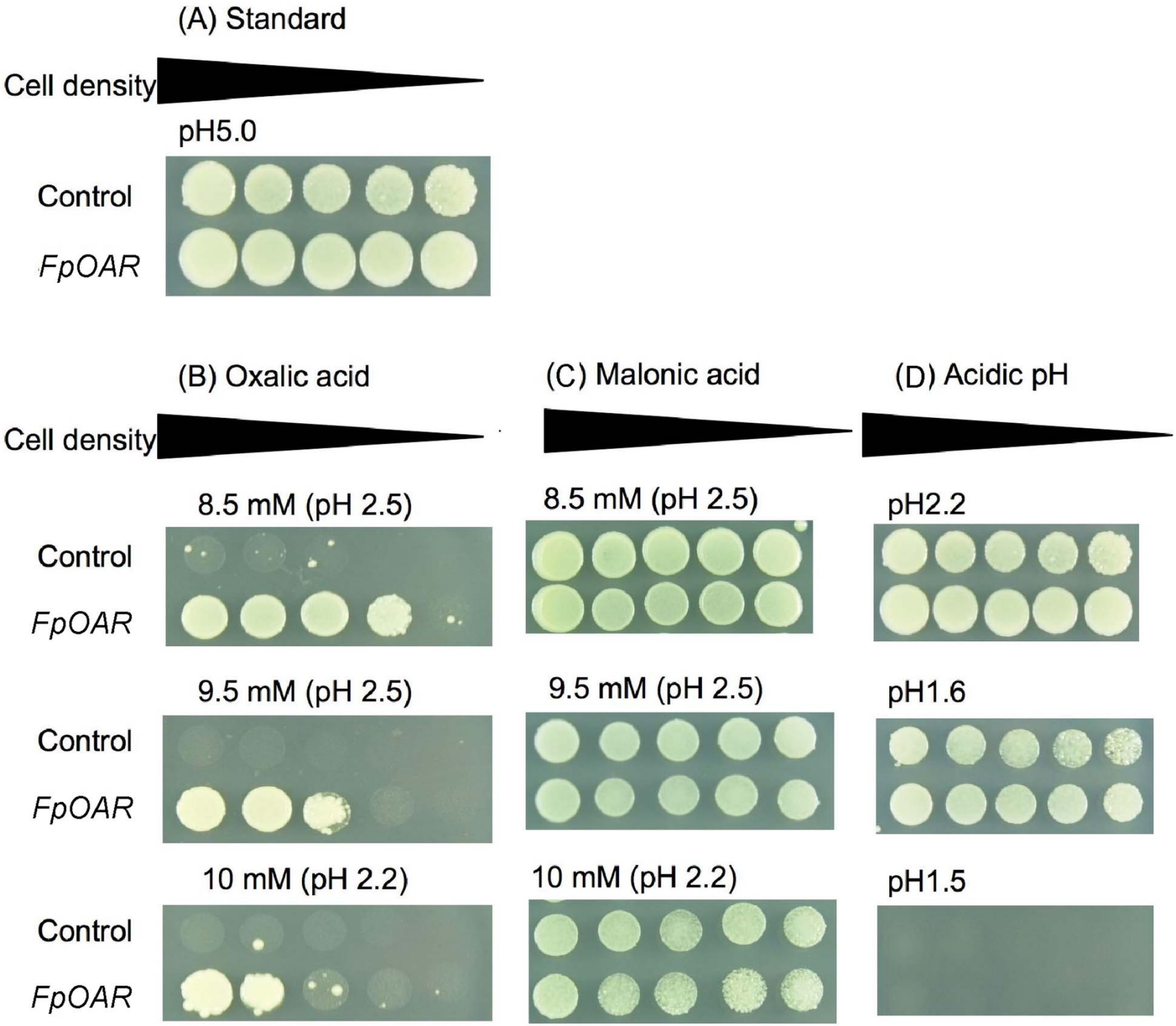
650 FIG. 7. Changes in (A) dry weight of mycelia, (B) oxalic acid accumulated and pH in
651 the medium during culture of *F. palustris*. In (C) the copy number is shown based on 0.2
652 μg total RNA, where the copy number is defined as the number of molecules of mRNA
653 coding FpOAR. The mean values and standard deviations were determined based on
654 triplicate samples. Mean value ±standard deviation.

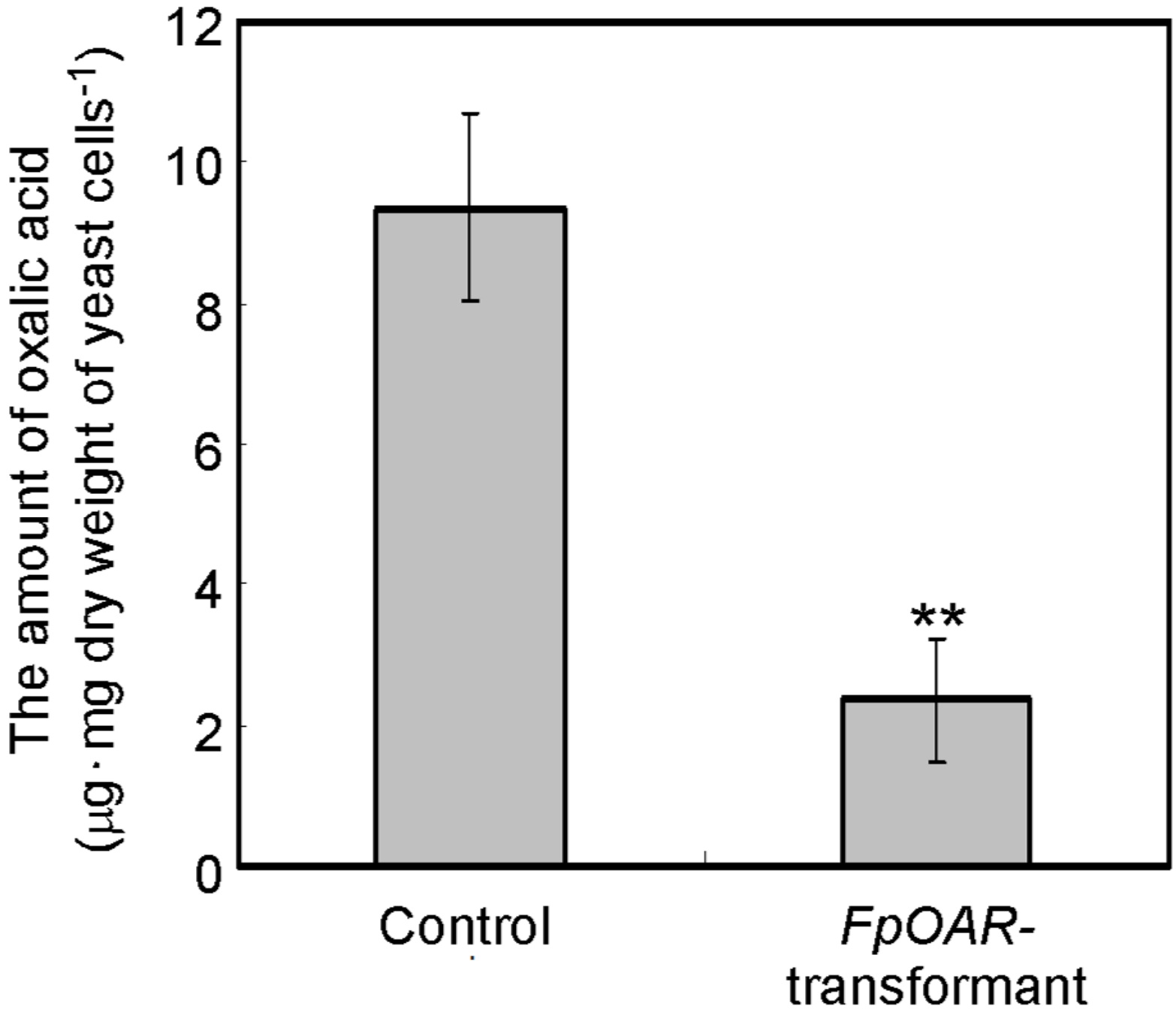
655

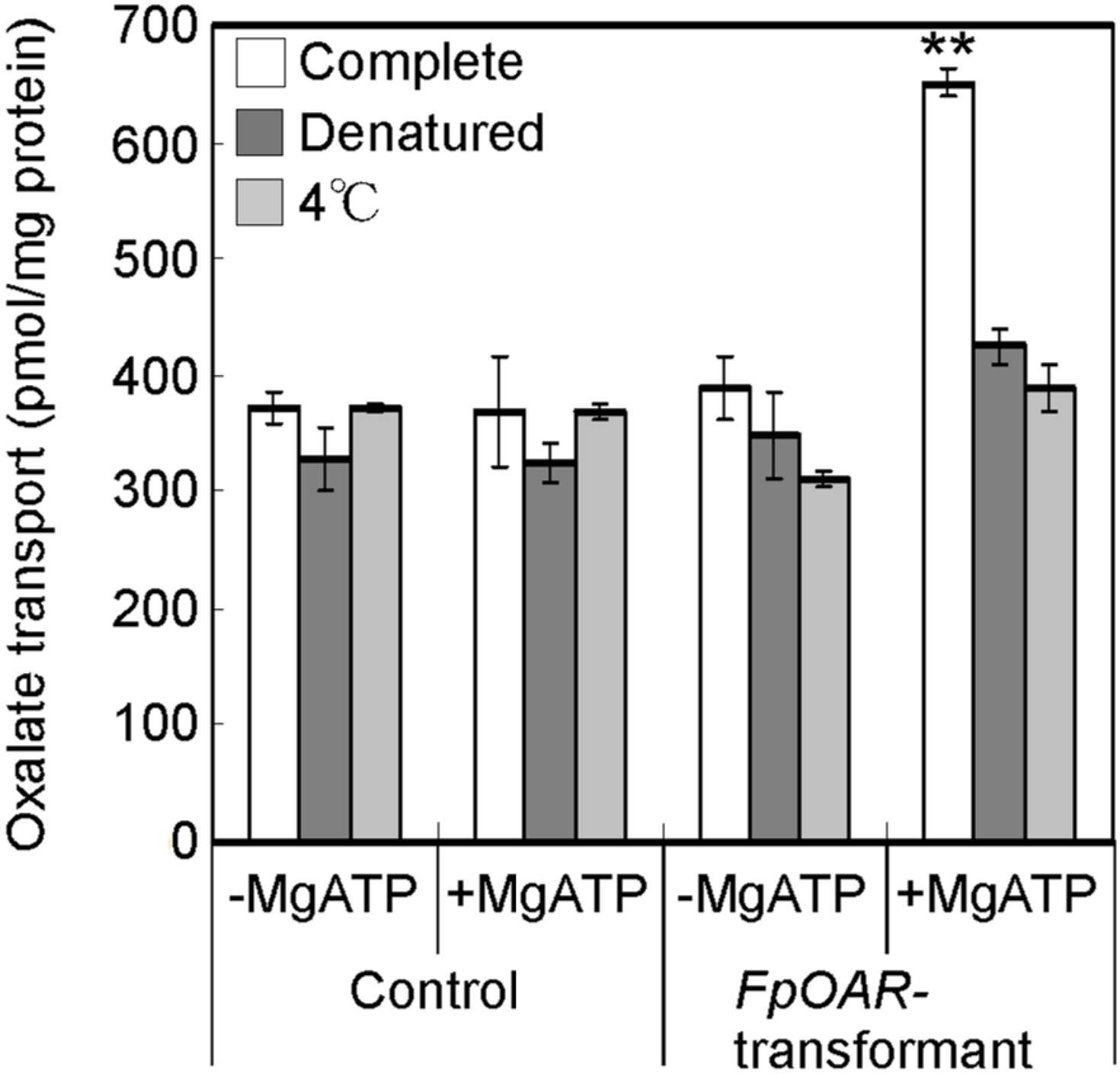
656 FIG. 8. Effect of exogenous addition of oxalic acid to the *F. palustris* culture on *FpOAR*
657 transcription. Mean value ±standard deviation. ** $p < 0.01$ ($n = 4$).

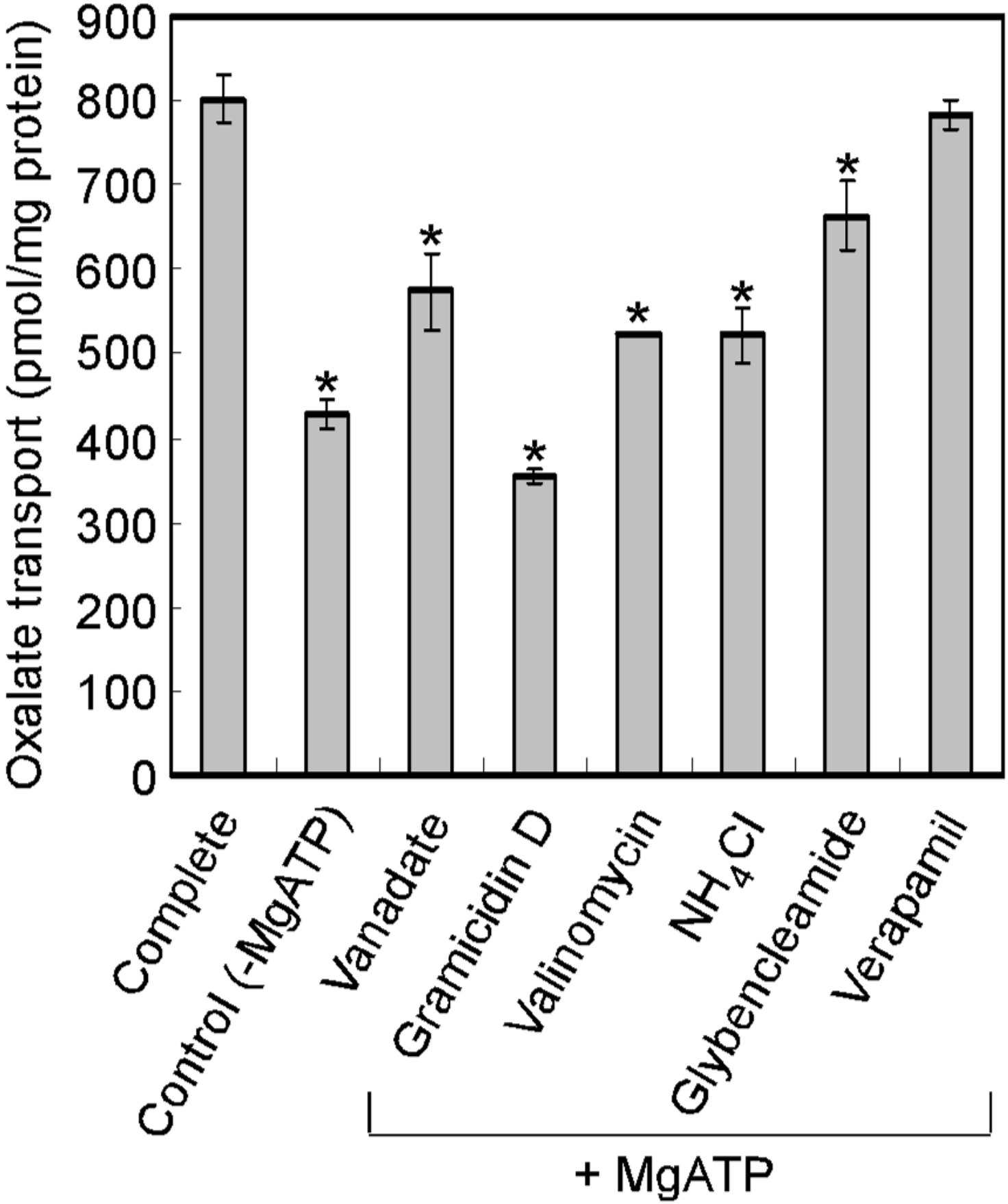

Oxalate transport (pmol/mg protein)
8000
7000
6000
5000
4000
3000
2000
1000
0
*
*
*
*
Complete
Control (-MgATP)
Vanadate
Valinomycin
NH4Cl
Glybencleamide
Cyclosporin A
+Mg ATP

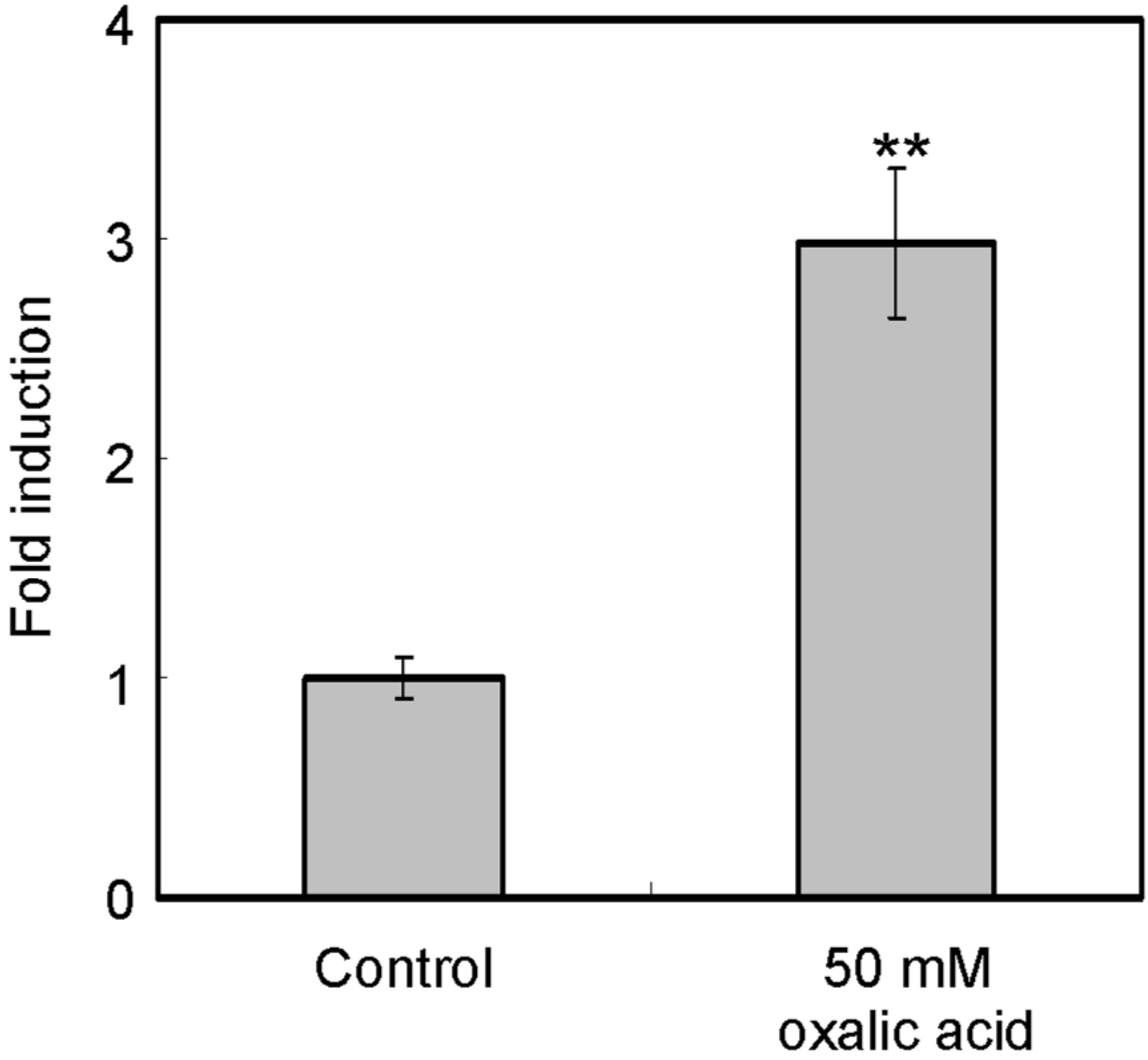

AAEY01000042
gwh2.7.329.1
605
Plus.C_740045
942
FpOAR
Lbscf0011g04660
0.05
976
AACS01000187

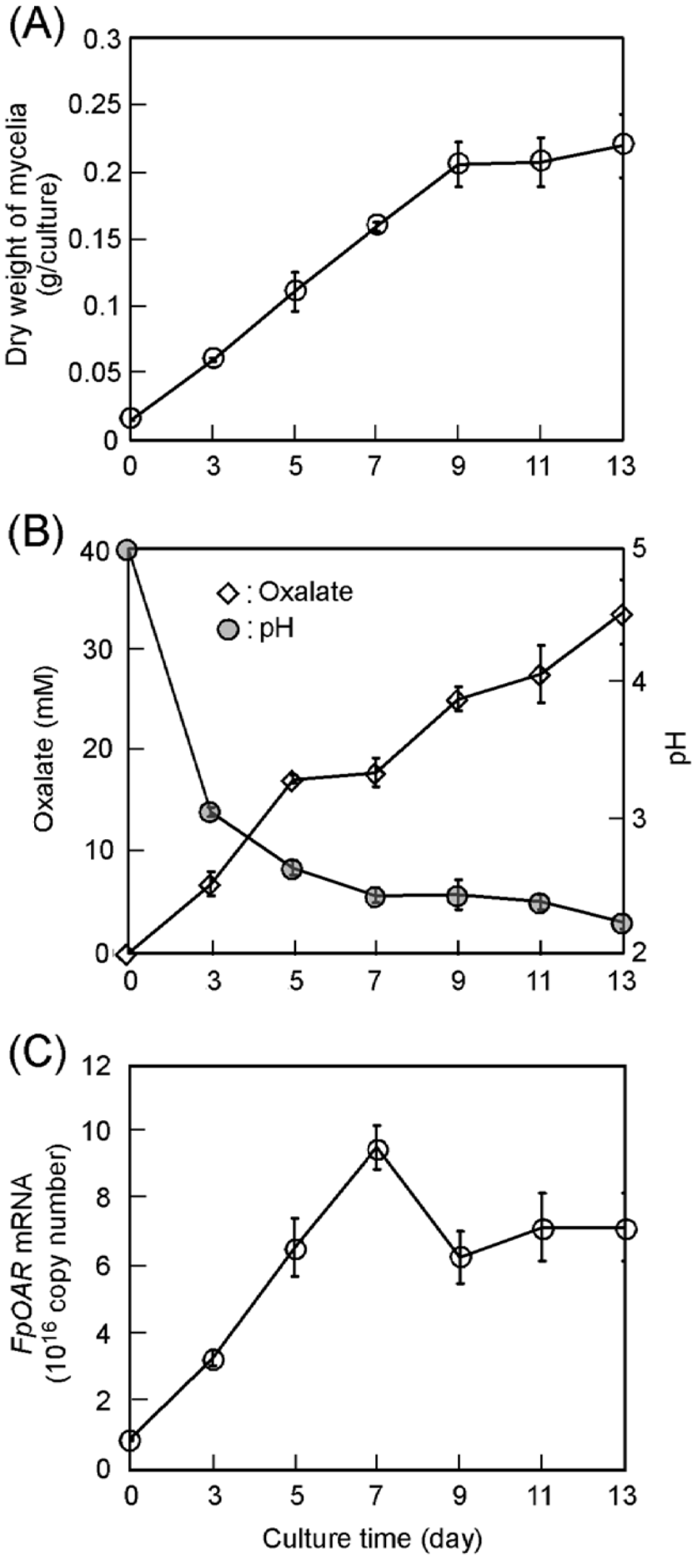

(A) Standard
Cell density
pH5.0
Control
FpOAR
(B) Oxalic acid
Cell density
8.5 mM (pH 2.5)
Control
FpOAR
9.5 mM (pH 2.5)
Control
FpOAR
10 mM (pH 2.2)
Control
FpOAR
(C) Malonic acid
8.5 mM (pH 2.5)
9.5 mM (pH 2.5)
10 mM (pH 2.2)
(D) Acidic pH
pH2.2
pH1.6
pH1.5


The amount of oxalic acid
(μg·mg dry weight of yeast cells-1)
12
10
8
6
4
2
0
**
Control
FpOAR-
transformant


Oxalate transport (pmol/mg protein)
700
600
500
400
300
200
100
0
Complete
Denatured
4℃
**
-MgATP
+MgATP
-MgATP
+MgATP
Control
FpOAR-
transformant


Oxalate transport (pmol/mg protein)
900
800
700
600
500
400
300
200
100
0
*
Complete
Control (-MgATP)
Vanadate
Gramicidin D
Valinomycin
NH4Cl
Glybencleamide
Verapamil
+ MgATP


4
3
2
1
0
Fold induction
**
Control
50 mM
oxalic acid


(A)
Dry weight of mycelia (g/culture)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
3
5
7
9
11
13
(B)
◇ : Oxalate
● : pH
Oxalate (mM)
pH
40
30
20
10
0
5
4
3
2
(C)
FpOAR mRNA (10^16 copy number)
12
10
8
6
4
2
0
Culture time (day)

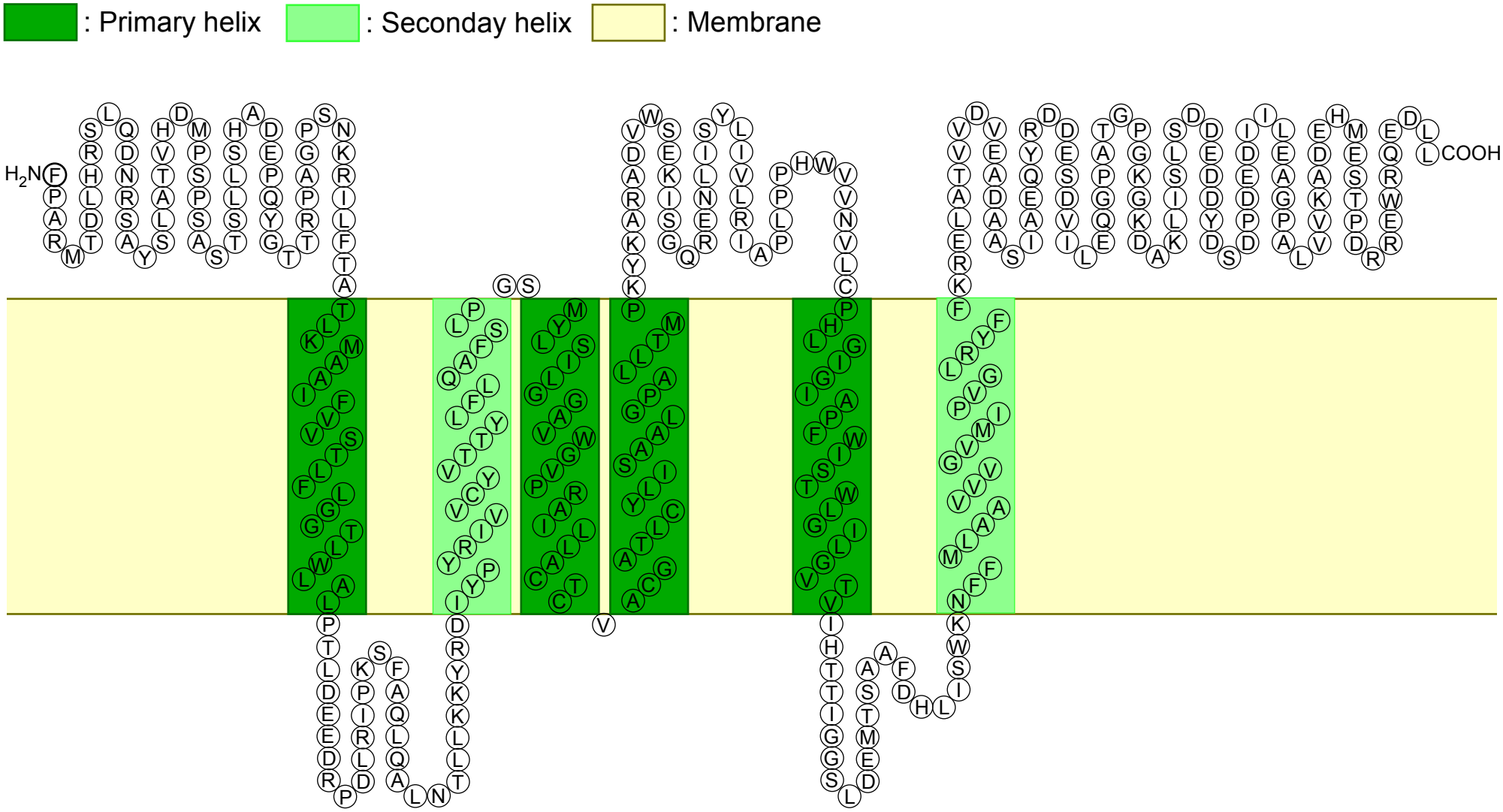


Fig. S1. Predicted transmembrane domains of FpOAR. The prediction was conducted with the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/). The predicted transmembrane domains are shown as primary and secondary helixes.

Supplemental Figure S1